Full Paper

Metagenomic analysis of bacterial communities on Dokdo Island

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Dokdo, located east of the mainland of South Korea, is a volcanic island designated as a natural monument of South Korea due to its ecological value. Dokdo is divided into Dongdo and Seodo, islands with geological differences. The soil bacterial communities on Dokdo (Dongdo and Seodo) were analyzed using the pyrosequencing method. There were 1,693 and 1,408 operational taxonomic units (OTU) from Dongdo and Seodo, respectively. The statistical analyses (rarefaction curves as well as Chao1, Shannon, and Simpson indices) showed that bacterial diversity was slightly higher in Dongdo than Seodo. From results of a BLASTN search against the Ez-Taxon-e database, the validated reads (obtained after sequence preprocessing) were almost all classified at the phylum level. From the phylum level down to the species level, the number of classified reads considerably decreased due to the absence of information concerning unculturable or unidentified bacteria to date. Among the 36 phyla identified, three phyla (Proteobacteria, Actinobacteria and Acidobacteria) accounted for around 74.64%. The taxonomic composition was similar at the higher ranks (family and above) between Dongdo and Seodo, but a little different at the genus level. There were also various differences in the relative abundance of taxonomic ranks between Dongdo and Seodo. In particular, the proportion of the genus *Acidobacterium* (of the phylum Acidobacteria) was about six times higher in Seodo than Dongdo. In ad-

dition, the percentage of the genus *Mycobacterium* (of the phylum Actinobacteria) was nearly three times higher in Seodo than Dongdo, and the proportion of the genus *Gaiella* was about 3.7 times higher in Dongdo than Seodo. Overall, through the metagenomic analysis, the number of species identified in Dongdo and Seodo was 1,239 and 1,055, respectively. This information on the numerous culturable and unculturable bacteria is expected to help in the screening of new species in Dokdo.

Key words: bacterial community; Dokdo Island; metagenomics

Introduction

Dokdo, located 217 km (37°14'12" N, 131°52'07" E) directly east of the mainland regions of Korea, is a volcanic island formed by lava eruptions (Kim et al., 2013; Sohn, 1995). This island has been preserved as a natural monument (since May 9, 2000) due to its unique ecological significance (<http://jikimi.cha.go.kr/english>). Because of frequent turbulent flow, there are typical oceanic climatic features in this region, with an average temperature of around 12°C and 85% air moisture throughout the year due to continuous rain or snow (Chang et al., 2002). Dokdo consists of two main islets, Dongdo and Seodo, but the whole island is one mass under the ocean. Dongdo is made up of andesitic volcanic rocks, while Seodo is composed of andesite and basalt rocks. Geographically, most of the land is covered under a 30 cm

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soil layer, and Dongdo and Seodo have a 60° steep slope (Sohn, 1995). Due to their poor soil layer, rainwater is immediately drained; consequently, these islets lack water. Thus, only a few (approximately 50–60) plant species exist on this island. In contrast, approximately 4,596 kinds of plant species exist on the mainland regions (Kim et al., 2007; Park et al., 2010; Shin et al., 2004).

The flora and fauna of Dokdo have been consistently studied, while the microflora has only been very recently researched. Between 2005 and 2013, 36 species of 4 phyla (Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes) were studied (National Center for Biotechnology Information (NCBI)). The phylum Firmicutes, a group of low-GC-content gram-positive bacteria, produces endospores. In this phylum, the new species *Virgibacillus dokdonensis* was identified for the first time, in 2005 (Yoon et al., 2005b). The phylum Proteobacteria, a group of gram-negative bacteria, includes various nitrogen fixers and some pathogens. This phylum is divided into the five classes: Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Gammaproteobacteria. The class Alphaproteobacteria includes various symbiotic bacteria of plants and animals. The new species *Porphyrobacter dokdonensis* and *Sphingomonas dokdonensis* of the class Alphaproteobacteria were identified (Yoon et al., 2006c, e). The new species *Variovorax dokdonensis* of Betaproteobacteria (which includes nitrogen fixers and pathogens) has also been newly identified (Yoon et al., 2006b). For the class Epsilonproteobacteria (the species of this class are known to inhabit the digestive tract of animals and serve as symbionts or pathogens), the species *Arcobacter marinus* has been reported (Kim et al., 2010). The new species *Lysobacter dokdonensis*, *Shewanella dokdonensis*, and *Stenotrophomonas dokdonensis* of the class Gammaproteobacteria (which includes various pathogens) were identified (Oh et al., 2011; Sung et al., 2012; Yoon et al., 2006d). The phylum Actinobacteria, a group of high-GC-content gram-positive bacteria, produces external spores. In this phylum, a total of nine species of six genera has been reported, and among the nine species, four (*Isoptericola dokdonensis*, *Nocardioides dokdonensis*, *Nocardioides hankookensis* and *Phycococcus dokdonensis*) are new species (Park et al., 2008; Yoon et al., 2006f, 2008a, b). The phylum Bacteroidetes is a group of gram-negative and anaerobic bacteria. Three new species (*Croceitalea dokdonensis*, *Maribacter dokdonensis*, and *Polaribacter dokdonensis*) of the class Flavobacteria have also been identified (Lee et al., 2008; Yoon et al., 2005a, 2006a).

To investigate these microflora, several methods, such as the culture-based assay, denaturing gradient gel electrophoresis (DGGE) and next generation sequencing (NGS)-based metagenomics, have been applied (Handelsman, 2004; Hong et al., 2011; Islam and Sar, 2011; Shokralla et al., 2012; Streit and Schmitz, 2004; Zhalnina et al., 2012). The culture-based assay has limitation in terms of the study of unculturable microbes from environmental samples, such as soil. Although the DGGE method can be applied to identify unculturable microbes, it also has limitation in terms of the acquisition of massive 16S rDNA sequences. Metagenomics (NGS-based technology) has been developed to study both culturable and unculturable microorganisms and for obtain-

ing massive sequence data (Handelsman, 2004; Shokralla et al., 2012). Among the NGS technologies, Roche 454 GS-FLX pyrosequencing is widely used in metagenomics (Gilles et al., 2011; Li et al., 2013). In this study, metagenomic analysis was performed using the GS-FLX pyrosequencing platform. Bacterial communities from soil samples of Dongdo and Seodo regions were analyzed and comparative analyses (bacterial composition and their relative abundance) were performed between Dongdo and Seodo samples.

Materials and Methods

Collection of soil samples. The sampling sites were in Dongdo (37°14'26.8" N, 131°52'10.4" E) and Seodo (37°14'30.6" N, 131°51'54.6" E) islets of Dokdo, Uljin-gun, Gyeongsangbuk-do, South Korea. The sample soils were collected by using cores (depth: 15 cm, diameter: 2 cm) at these sites and were homogenized separately for experiments.

DNA extraction and pyrosequencing. For pyrosequencing analysis, metagenomic DNA was extracted from soil samples with the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). The resulting DNA was amplified using barcoded fusion primers targeting the V1/V3 regions of 16S rRNA genes (Wang and Qian, 2009). The fusion primers contained 454 specific adapters, a key sequence (4 bp), a barcode (7–11 bp unique sequence), a linker sequence (2 bp), and universal primers (518R and 27F). The forward primer sequence was 5'-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-GAGTTTGATCMTGGCTCAG-3' and the reverse primer sequence was 5'-CCA TCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-WTTACCGCGGTGCTGG-3'; the "X", representing the barcode sequence, was used to separate samples. The PCR reaction was carried out using the PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA). All PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and the DNA products were quantified using both the QuantiTTM PicoGreen[®] dsDNA Assay Kit (Invitrogen, Carlsbad, CA) and the TBS-380 Mini Fluorometer (Turner BioSystems, Sunnyvale, CA). The same amounts of the resulting PCR products from each sample were pooled and electrophoresed to select the DNA samples longer than 300 bp. The length of DNA fragments was checked again by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) (Panaro et al., 2000). The pyrosequencing was performed by Chunlab, Inc. (Seoul, South Korea) using the 454 GS FLX Titanium Sequencing System (Roche, Branford, CT). All pyrosequencing data were submitted to the EMBL Sequence Read Archive (SRA) database under the study accession number PRJEB4517 (<http://www.ebi.ac.uk/ena/data/view/PRJEB4517>).

Taxonomical identification and statistical data analysis. All preprocessed reads were identified by performing a BLASTN search against the EzTaxon-e database (Altschul et al., 1990; Kim et al., 2012). For the classification, the following rules were applied: 1) when the results could not be classified into a sublevel, "uc" was added at the end of name, 2) if the taxonomic name was unknown, the genus name was written first and the initial letter of each unknown level was written at the end of the name (e.g., if the family

name was unknown, an “f” was written after the genus name. “Devosia_F”; c=class, o=order, f=family). The following cutoff values were used for taxonomic assignment: species ($x \geq 97\%$), genus ($97\% > x \geq 94\%$), family ($94\% > x \geq 90\%$), order ($90\% > x \geq 85\%$), class ($85\% > x \geq 80\%$), and phylum ($80\% > x \geq 75\%$) (Chun et al., 2007; Park et al., 2012).

All statistical analyses of bacterial communities were performed using CLcommunity software (Chunlab, Inc., Seoul, South Korea). Operational Taxonomic Units (OTUs) were analyzed with the CD-HIT program at a 97% sequence similarity (Li and Godzik, 2006). The Mothur platform was used for calculating rarefaction curves and diversity indices (Chao1, Shannon, and Simpson) (Heck et al., 1975; Schloss et al., 2009).

Results and Discussion

Pyrosequencing results and statistical analyses

A total of 7,683 reads was obtained from the soil sample from Dongdo, and after preprocessing of the sequences, the number of validated reads was 3,239. The mean read length of the resulting sequences was 461.81 bp and the maximum read length was 525 bp. From Seodo, a total of 5,740 reads was obtained, and the number of validated reads was 2,700. The mean read length and maximum read length of the resulting reads were 462.89 bp and 517 bp, respectively. The number of OTUs was 1,693 and 1,408 from Dongdo and Seodo, respectively, at a 97% similarity level (Table 1). The rarefaction curves showed that species richness in Dongdo was slightly higher than in Seodo (Fig. 1). The Chao1 (species richness estimation) indicated that species richness was relatively higher in Dongdo (3,975) than Seodo (3,455). The Shannon and Simpson diversity indices showed that Dongdo (Shannon: 7.03; Simpson: 0.0013) had a more diverse bacterial community compared with Seodo (6.79; 0.0022) (Table 1). However, we inferred that these differ-

Table 1. Pyrosequencing results and statistical analyses.

	Dongdo	Seodo
Number of total reads	7,683	5,740
Number of validated reads	3,239	2,700
Mean read length (bp)	461.81	462.89
Maximum read length (bp)	525	517
Number of OTUs ^a	1,693	1,408
Chao1 ^b	3,975	3,455
Shannon ^c	7.03	6.79
Simpson ^d	0.0013	0.0022
Good's coverage ^e	0.648	0.647

The number of total pyrosequencing reads was 13,423 and the total validated reads for taxonomic assignments was 5,939. The results of statistical analyses for bacterial communities show that Dongdo has higher OTUs, species richness, and diversity compared to Seodo.

^aOTUs: Operational Taxonomic Units. ^bChao1: Species richness estimation. ^cShannon: Shannon diversity index (>0 , higher, more diverse). ^dSimpson: Simpson diversity index (0–1, 1=most simple). ^eGood's coverage: $1 - (\text{number of singleton OTUs} / \text{number of sequences})$, 1=100% coverage.

ences (in the number of OTUs, rarefaction curves, and statistical analyses) between Dongdo and Seodo were affected by different numbers of validated reads. Furthermore, the numerical values of Good's coverage were not high in either Dongdo (0.648) or Seodo (0.647). Therefore, additional sequencing is required to obtain a more accurate comparison of the bacterial diversity between Dongdo and Seodo.

Analysis of the soil bacterial community on Dokdo

As the result of a BLASTN search against the EzTaxon-e database, each validated read was assigned a bacterial taxonomy (Table 2). When the BLASTN hit had the scientific name (phylum, class, order, family, genus, and species names) for the taxon, it was designated as “classified”. On the other hand, when the hit had an unknown name, includ-

Fig. 1. Rarefaction curves for Operational Taxonomic Units (OTUs) from Dongdo and Seodo samples.

OTUs were clustered at 3% dissimilarity using CD-HIT. The microbial community in Dongdo (OTUs, 1,693; sequencing reads, 3,239) had more OTUs and reads than that in Seodo (1,408; 2,700).

Table 2. Number of bacterial taxa observed in Dongdo and Seodo.

	Dongdo		Seodo	
	c ^a	uc ^b	c ^a	uc ^b
Phylum	3,232	7	2,688	12
Class	2,780	459	2,469	231
Order	2,579	660	2,297	403
Family	2,097	1,142	1,986	714
Genus	1,251	1,988	1,235	1,465
Species	361	2,878	381	2,319

The number of unclassified sequences increases greatly towards taxon sublevel. In terms of total bacterial sequences, the percentage of unclassified species was 0.89% and 0.86% for Dongdo and Seodo samples, respectively.

^aNumber of sequencing reads classified into scientific name for taxon. ^bNumber of sequencing reads either unclassified into sublevel or classified into unknown name for taxon.

ing a name composed of the GenBank accession number, it was designated as “unclassified”. For sample from Dongdo, 3,232 reads were classified at the phylum level, except for seven reads. A total of 2,780 sequences were classified at the class level, but 459 sequences were not. At the order and family levels, 2,579 and 2,097 reads were classified, and 660 and 1,142 sequences were unclassified, respectively. Among a total of 3,239 validated reads, only 361 sequences were

classified at the species level. For the sample from Seodo, 2,688 reads were classified at the phylum level, but 12 reads were unclassified. Except for 231 reads, 2,469 sequences were classified at the class level. A total of 2,297 reads were classified at the order level, but 403 reads were not. At the family and genus levels, 1,986 and 1,235 reads were classified, respectively. Among 2,700 reads, only 381 reads were classified at the species level. For both the Dongdo and Seodo samples, the number of classified reads decreased from the phylum level down to the genus level. Up to now, about 99.8% of microbes on Earth have not been cultured (Amann et al., 1995); thus, most of the reads were not classified at the species level. The culture-based approaches or DGGE have limits in the identification of unculturable microbes and for performing massive 16S rRNA sequencing. Therefore, metagenomics using the pyrosequencing method is a powerful tool for the identification of unculturable microbes. Above all, metagenomic analyses are able to provide useful information on new species that exist in the natural environment of Dokdo.

The taxonomic composition in Dokdo (Dongdo and Seodo) was analyzed, and a total of 36 phyla was identified. Among these 36 phyla, 17 phyla had scientific names for taxa, namely Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Chlorobi, Chloroflexi, Cyanobacteria,

Fig. 2. Taxonomic composition of bacterial phyla from Dongdo and Seodo samples.

Bacterial phyla are only shown for phyla with a relative abundance of more than 1% in either Dongdo or Seodo. Three phyla were dominant (74.64% of total phyla) and shared between the two sites: Proteobacteria, Actinobacteria, and Acidobacteria.

Deinococcus-Thermus, Elusimicrobia, Fibrobacteres, Firmicutes, Gemmatimonadetes, Nitrospirae, Planctomycetes, Proteobacteria, Tenericutes, and Verrucomicrobia. Three phyla (Acidobacteria, Actinobacteria, and Proteobacteria) accounted for approximately 74.64% of the total phyla. Thirteen other phyla (namely 10BAV, AD3, BRC1, GN02, MATCR, NKB19, OD1, OP11, OP3, TM6, TM7, WS3, and WS5) were candidate divisions. One phylum was Bacteria_uc, which belongs to the kingdom Bacteria but does not have an exact phylum. The remaining five phyla (AF234118_p, CU922841_p, DQ404828_p, EF688356_p, and JX172748_p) were designated according to GenBank accession number. As mentioned above, only 742 reads (for Dongdo and Seodo samples) had species names. However, in contrast, most sequences (97.05%) were classified under phyla with scientific names for taxa. In our study, biological classification (from the phylum to the species level) for the read was analyzed. Based on these results, most unclassified bacteria at the species level can be potentially classified with information on higher taxonomic levels (the phylum, class, order, family, and genus).

Comparison of bacterial communities between Dongdo and Seodo

The relative abundance of bacterial communities, at the phylum level, was analyzed between the Dongdo and Seodo samples (Fig. 2). Proteobacteria was the dominant phylum for both the Dongdo (32.79%) and Seodo (35.81%) samples. There were considerable differences in the proportion of four phyla between Dongdo and Seodo. The relative abundance of Actinobacteria was high in Dongdo (30.07%), compared with Seodo (17.78%). In addition, the proportion of Cyanobacteria was significantly higher in Dongdo (2.50%) than in Seodo (0.30%), and the percentage of Nitrospirae in Dongdo (1.17%) was higher than in Seodo (0.52%). On the other hand, the proportion of Bacteroidetes in Seodo (9.63%) was approximately 2.48 times higher than in Dongdo (3.89%). Taxonomic composition was also analyzed at the class level between Dongdo and Seodo. The classes (accounting for at least 1% of either the Dongdo or Seodo samples and having a scientific name), were assessed (Fig. 3). In the phylum Acidobacteria, the classes Acidobacteria_c (Dongdo, 4.91%; Seodo, 8.26%) and Chloracidobacterium_c (2.04%; 4.19%) were more abundant in Seodo than Dongdo. In contrast, the class Solibacteres (3.30%; 1.85%) was more abundant in Dongdo than Seodo.

Fig. 3. Taxonomic composition of bacterial classes from Dongdo and Seodo samples.

Bacterial classes are only shown for classes with a relative abundance of more than 1% in either Dongdo or Seodo. The 19 classes of eight phyla (Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Planctomycetes, and Proteobacteria) are shown.

Table 3. Relative abundance of bacterial orders in Dongdo and Seodo.

Taxonomy	Dongdo		Seodo	
	% ^a	Fr ^b	% ^a	Fr ^b
Acidobacteria_Acidobacteria_c_Acidobacteriales	4.91	159	8.26	223
Acidobacteria_Chloracidobacterium_c_Blastocatella_o	1.45	47	3.85	104
Acidobacteria_Solibacteres_Solibacterales	2.32	75	1.85	50
Actinobacteria_Acidimicrobiia_Acidimicrobiales	2.69	87	1.33	36
Actinobacteria_Actinobacteria_c_Corynebacteriales	2.04	66	5.22	141
Actinobacteria_Actinobacteria_c_Frankiales	1.88	61	1.70	46
Actinobacteria_Actinobacteria_c_Micrococcales	0.86	28	2.19	59
Actinobacteria_Actinobacteria_c_Micromonosporales	2.13	69	0.96	26
Actinobacteria_Actinobacteria_c_Propionibacteriales	3.33	108	1.81	49
Actinobacteria_Actinobacteria_c_Pseudonocardiales	2.19	71	0.56	15
Actinobacteria_Actinobacteria_c_Streptomycetales	1.36	44	0.37	10
Actinobacteria_Actinobacteria_c_Streptosporangiales	2.96	96	0.56	15
Actinobacteria_Rubrobacteria_Gaiellales	2.99	97	0.78	21
Actinobacteria_Thermoleophilia_Solirubrobacteriales	5.71	185	1.56	42
Bacteroidetes_Cytophagia_Cytophagales	0.90	29	2.00	54
Bacteroidetes_Flavobacteria_Flavobacteriales	0.28	9	2.26	61
Bacteroidetes_Sphingobacteria_Sphingobacteriales	2.32	75	4.96	134
Firmicutes_Bacilli_Bacillales	0.71	23	2.63	71
Gemmatimonadetes_Gemmatimonadetes_c_Gemmatimonadales	1.88	61	2.59	70
Planctomycetes_Planctomycetacia_Planctomycetales	1.70	55	2.19	59
Proteobacteria_Alphaproteobacteria_Rhizobiales	12.26	397	8.74	236
Proteobacteria_Alphaproteobacteria_Rhodospirillales	4.82	156	2.96	80
Proteobacteria_Alphaproteobacteria_Sphingomonadales	0.96	31	1.78	48
Proteobacteria_Betaproteobacteria_Burkholderiales	2.16	70	2.78	75
Proteobacteria_Deltaproteobacteria_Myxococcales	1.39	45	2.44	66
Proteobacteria_Gammaproteobacteria_Steroidobacter_o	2.93	95	2.11	57
Proteobacteria_Gammaproteobacteria_Xanthomonadales	3.27	106	7.26	196
Total	72.40	2,345	75.70	2,044

The list of bacterial orders is only shown for orders with a relative abundance of more than 1% in either Dongdo or Seodo. Taxon names (phylum, class, and order) are concatenated using an underline ().

^aRelative abundance. ^bFrequency of bacteria detected in each sampling site.

The four classes in the phylum Actinobacteria were Acidimicrobiia (2.69%; 1.33%), Actinobacteria_c (17.69%; 13.70%), Rubrobacteria (3.27%; 1.11%), and Thermoleophilia (5.71%; 1.56%). The percentages of these four classes were higher in Dongdo compared to Seodo. The three classes in the phylum Bacteroidetes, Cytophagia (0.90%; 2.00%), Flavobacteria (0.28%; 2.26%), and Sphingobacteria (2.59%; 5.37%), were more abundant in Seodo than Dongdo. The two classes in the phylum Chloroflexi, Anaerolineae (0.68%; 1.26%) and Chloroflexi_c (0.31%; 1.11%), were relatively higher in abundance in Seodo compared to Dongdo. Three classes in the phylum Proteobacteria, Betaproteobacteria (3.52%; 4.93%), Deltaproteobacteria (2.72%; 3.52%), and Gammaproteobacteria (7.19%; 12.07%), were more abundant in Seodo than Dongdo. However, one class in the phylum Proteobacteria, Alphaproteobacteria (19.30%; 15.19%), was more abundant in Dongdo than Seodo.

The taxonomic composition of 27 orders (accounting for at least 1% in either Dongdo or Seodo and having a scientific name) was analyzed (Table 3). In the phylum Acidobacteria, two orders Acidobacteriales (4.91%; 8.26%) and Blastocatella_o (1.45%; 3.85%) were more abundant in Seodo than Dongdo, whereas the order Solibacteriales (2.32%; 1.85%) was more abundant in Dongdo than Seodo. In contrast, the relative abundance of nine orders in the phylum Actinobacteria was higher in Dongdo than Seodo, except for Corynebacteriales (2.04%; 5.22%) and Micrococcales (0.86%; 2.19%). In particular, the proportion of the

order Solirubrobacteriales in Dongdo (5.71%) was about 3.67 times higher than its proportion in Seodo (1.56%). The three orders in the phylum Bacteroidetes were more abundant in Seodo than Dongdo. For the class Alphaproteobacteria in the phylum Proteobacteria, the proportion of two orders Rhizobiales and Rhodospirillales were higher in Dongdo than Seodo, but not that of the order Sphingomonadales. The relative abundance of two orders in two classes (Betaproteobacteria and Deltaproteobacteria) was more abundant in Seodo than Dongdo. The order Steroidobacter_o in the class Gammaproteobacteria was more abundant in Dongdo than Seodo; in contrast, the order Xanthomonadales was more abundant in Seodo than Dongdo.

The relative abundance of 28 families (accounting for at least 1% in either Dongdo or Seodo and having a scientific name) was analyzed (Table 4). Two families in the phylum Acidobacteria, Acidobacteriaceae (4.72%; 8.22%) and Blastocatella_f (1.11%; 1.52%), were more abundant in Seodo than Dongdo. The proportion of the family Solibacteraceae (1.82%; 1.81%) was similar between Dongdo and Seodo. The relative abundance of nine families in the phylum Acidobacteria was higher in Dongdo compared to Seodo, but not that of the family Mycobacteriaceae. Three families in the phylum Bacteroidetes were more abundant in Seodo than Dongdo. For the class Alphaproteobacteria in the phylum Proteobacteria, three families (Bradyrhizobiaceae, Hyphomicrobiaceae, and Acetobacteraceae) were more abundant in Dongdo than Seodo. In contrast, three other

Table 4. Relative abundance of bacterial families in Dongdo and Seodo.

Taxonomy	Dongdo		Seodo	
	% ^a	Fr ^b	% ^a	Fr ^b
Acidobacteria_Acidobacteria_c_Acidobacteriales_Acidobacteriaceae	4.72	153	8.22	222
Acidobacteria_Chloracidobacterium_c_Blastocatella_o_Blastocatella_f	1.11	36	1.52	41
Acidobacteria_Solibacteres_Solibacterales_Solibacteraceae	1.82	59	1.81	49
Actinobacteria_Acidimicrobiia_Acidimicrobiales_Acidimicrobiaceae	1.14	37	0.30	8
Actinobacteria_Actinobacteria_c_Corynebacteriales_Mycobacteriaceae	1.48	48	4.67	126
Actinobacteria_Actinobacteria_c_Frankiales_Frankiaceae	1.02	33	0.74	20
Actinobacteria_Actinobacteria_c_Micromonosporales_Micromonosporaceae	2.13	69	0.96	26
Actinobacteria_Actinobacteria_c_Propionibacteriales_Nocardiodaceae	2.13	69	1.33	36
Actinobacteria_Actinobacteria_c_Propionibacteriales_Propionibacteriaceae	1.20	39	0.48	13
Actinobacteria_Actinobacteria_c_Pseudonocardiales_Pseudonocardaceae	2.19	71	0.52	14
Actinobacteria_Actinobacteria_c_Streptomycetales_Streptomycetaceae	1.36	44	0.37	10
Actinobacteria_Rubrobacteria_Gaiellales_Gaiellaceae	2.87	93	0.78	21
Actinobacteria_Thermoleophilia_Solirubrobacterales_Conexibacteraceae	1.64	53	0.33	9
Bacteroidetes_Cytophagia_Cytophagales_Cytophagaceae	0.52	17	1.04	28
Bacteroidetes_Flavobacteria_Flavobacteriales_Flavobacteriaceae	0.25	8	2.07	56
Bacteroidetes_Sphingobacteria_Sphingobacteriales_Chitinophagaceae	2.10	68	4.22	114
Firmicutes_Bacilli_Bacillales_Planococcaceae	0.03	1	1.07	29
Gemmatimonadetes_Gemmatimonadetes_c_Gemmatimonadales_Gemmatimonadaceae	1.17	38	2.04	55
Planctomycetes_Planctomycetacia_Planctomycetales_Planctomycetaceae	1.54	50	2.07	56
Proteobacteria_Alphaproteobacteria_Rhizobiales_Bradyrhizobiaceae	5.68	184	3.44	93
Proteobacteria_Alphaproteobacteria_Rhizobiales_Devosia_f	0.46	15	1.22	33
Proteobacteria_Alphaproteobacteria_Rhizobiales_Hyphomicrobiaceae	2.53	82	0.81	22
Proteobacteria_Alphaproteobacteria_Rhizobiales_Rhizomicrobium_f	1.02	33	1.33	36
Proteobacteria_Alphaproteobacteria_Rhodospirillales_Acetobacteraceae	1.82	59	0.89	24
Proteobacteria_Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae	0.49	16	1.15	31
Proteobacteria_Deltaproteobacteria_Myxococcales_Haliangiaceae	0.62	20	1.00	27
Proteobacteria_Gammaproteobacteria_Steroidobacter_o_Steroidobacter_f	2.32	75	1.33	36
Proteobacteria_Gammaproteobacteria_Xanthomonadales_Xanthomonadaceae	3.27	106	7.11	192
Total	48.66	1,576	52.85	1,427

The list of bacterial families is only shown for families with a relative abundance of more than 1% in either Dongdo or Seodo. Taxon names (phylum, class, order, and family) are concatenated using an underline (_).

^aRelative abundance. ^bFrequency of bacteria detected in each sampling site.

families (*Devosia_f*, *Rhizomicrobium_f* and *Sphingomonadaceae*) were more abundant in Seodo than Dongdo. The proportion of the family *Haliangiaceae* in the class *Deltaproteobacteria* was higher in Seodo than Dongdo. The family *Xanthomonadaceae* in the class *Gammaproteobacteria* was also higher in Seodo compared to Dongdo.

The taxonomic composition of 75 genera (having a scientific name) was analyzed (data not shown). Among them, 20 genera, showing considerable differences in proportion between Dongdo and Seodo samples, were assessed (Table 5). For the phylum *Acidobacteria*, in particular, the proportion of the genus *Acidobacterium* in Seodo was approximately six times higher than in Dongdo. In the phylum *Actinobacteria*, seven genera were more abundant in Dongdo than Seodo; in particular, the proportion of the genus *Gaiella* was approximately 3.7 times higher in Dongdo than Seodo. The percentage of the genus *Mycobacterium*, however, was about three times higher in Seodo than Dongdo. Two genera, *Flavobacterium* and *Subsaxibacter*, in the phylum *Bacteroidetes* were more abundant in Seodo than Dongdo. The proportion of the genus *Sporosarcina* in the phylum *Firmicutes* was also higher in Seodo than Dongdo. For the class *Alphaproteobacteria* in the phylum *Proteobacteria*, three genera, excluding the genus *Devosia*, were more abundant in Dongdo than Seodo. The proportions of two genera, *Steroidobacter* and *Rhodanobacter*, in the class *Gammaproteobacteria* were higher in Seodo than Dongdo.

The taxonomic composition at higher levels (phylum, class, order, family) was very similar between the Dongdo and Seodo samples. However, at the genus level, the composition of taxa showed slight differences. In particular, there was a six-fold difference in the percentage of the genus *Acidobacterium*. However, most of *Acidobacterium* species identified in this study did not have a scientific name for their taxon. In addition, few studies on the physiological characteristics of the genus *Acidobacterium* have been conducted. Thus, we were unable to infer the cause of these differences between the Dongdo and Seodo samples. It was also difficult to explain the causes for differences in the relative abundance of other species between Dongdo and Seodo. As mentioned earlier, the numerical values of Good's coverage were slightly low for both Dongdo and Seodo samples. To analyze the relative abundance of species between Dongdo and Seodo more precisely, metagenomic analyses from other locations are required.

By using molecular identification based on 16S rRNA, Janssen confirmed that nine bacterial phyla (*Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes*) are generally dominant in a soil environment (Janssen, 2006). In previous studies on soil bacteria communities identified through the pyrosequencing method, three major phyla, *Acidobacteria*, *Actinobacteria*, and *Proteobacteria*, were also detected worldwide: in the

Table 5. Discriminative bacterial genera between Dongdo and Seodo.

Taxonomy	Dongdo		Seodo	
	% ^a	Fr ^b	% ^a	Fr ^b
Acidobacteria_Acidobacteria_c_Acidobacteriales_Acidobacteriaceae_Acidobacterium	0.99	32	6.04	163
Acidobacteria_Acidobacteria_c_Acidobacteriales_Acidobacteriaceae_Bryocella	0.37	12	0.00	0
Acidobacteria_Acidobacteria_c_Acidobacteriales_Acidobacteriaceae_Granulicella	0.74	24	0.11	3
Actinobacteria_Acidimicrobiia_Acidimicrobiales_Acidimicrobiaceae_Aciditerrimonas	0.80	26	0.15	4
Actinobacteria_Actinobacteria_c_Corynebacteriales_Mycobacteriaceae_Mycobacterium	1.48	48	4.52	122
Actinobacteria_Actinobacteria_c_Propionibacteriales_Nocardiodaceae_Marmoricola	0.56	18	0.11	3
Actinobacteria_Actinobacteria_c_Propionibacteriales_Propionibacteriaceae_Microlunatus	1.11	36	0.22	6
Actinobacteria_Actinobacteria_c_Pseudonocardiales_Pseudonocardiaceae_Pseudonocardia	0.65	21	0.15	4
Actinobacteria_Actinobacteria_c_Streptomycetales_Streptomycetaceae_Streptomyces	1.23	40	0.37	10
Actinobacteria_Rubrobacteria_Gaiellales_Gaiellaceae_Gaiella	2.78	90	0.74	20
Actinobacteria_Thermoleophilia_Solirubrobacterales_Conexibacteraceae_Conexibacter	1.57	51	0.33	9
Bacteroidetes_Flavobacteria_Flavobacteriales_Flavobacteriaceae_Flavobacterium	0.19	6	0.74	20
Bacteroidetes_Flavobacteria_Flavobacteriales_Flavobacteriaceae_Subtaxibacter	0.00	0	0.44	12
Firmicutes_Bacilli_Bacillales_Planococcaceae_Sporosarcina	0.00	0	1.04	28
Proteobacteria_Alphaproteobacteria_Rhizobiales_Bradyrhizobiaceae_Bradyrhizobium	1.08	35	0.33	9
Proteobacteria_Alphaproteobacteria_Rhizobiales_Bradyrhizobiaceae_Pseudolabrys	2.07	67	0.85	23
Proteobacteria_Alphaproteobacteria_Rhizobiales_Devosia_f_Devosia	0.40	13	1.11	30
Proteobacteria_Alphaproteobacteria_Rhizobiales_Hyphomicrobiaceae_Methylobacter	0.77	25	0.15	4
Proteobacteria_Gammaproteobacteria_Steroidobacter_o_Steroidobacter_f_Steroidobacter	0.15	5	0.56	15
Proteobacteria_Gammaproteobacteria_Xanthomonadales_Xanthomonadaceae_Rhodanobacter	0.12	4	0.70	19
Total	17.07	553	18.67	504

A total of 75 genera were classified into scientific names for taxon. There is a considerable difference in 20 genera between Dongdo and Seodo; Seodo has more than six times the members of the genus *Acidobacterium* and more than three times the members of the genus *Mycobacterium* than Dongdo. Taxon names (phylum, class, order, family, and genus) are concatenated using an underline (_).

^a Relative abundance. ^b Frequency of bacteria detected in each sampling site

Americas (e.g., Brazil, Canada, Florida, and Illinois) (Roesch et al., 2007), in German forests and grasslands (Nacke et al., 2011), and in forests in China, Japan, and Malaysia (Miyashita et al., 2013; Singh et al., 2013; Singh et al., 2012; Tripathi et al., 2012).

Correspondingly, overall major phyla in Dokdo were Proteobacteria, Actinobacteria, and Acidobacteria with an average relative abundance of 34.3%, 23.9%, and 16.1%, respectively. However, the composition of bacterial phyla was somewhat different between Dongdo and Seodo; for example, Proteobacteria and Actinobacteria were dominant at a similar proportion (about 30%) in Dongdo, whereas the abundance of Proteobacteria (35.81%) was about two times higher than that of Actinobacteria (17.78%) in Seodo.

On the other hand, a pyrosequencing study on microbial communities on the coasts of Sichang Island (Thailand) showed that Proteobacteria (over 50%), Actinobacteria (about 30%), and Bacteroidetes (about 10%) are dominant bacterial phyla. The abundance of Bacteroidetes on Sichang Island was similar to that on Seodo (9.63%), but not Dongdo (3.89%). In particular, Acidobacteria on Sichang Island was at a very low proportion (less than 0.1%) compared to that on Dokdo (about 16.1%) (Somboonna et al., 2012).

Soil bacterial communities can be defined by various environment factors. Dokdo, a rocky island, is covered with few plant species and is affected by its marine environment. Thus, these unique environment factors may affect the soil bacterial communities on Dokdo. However, compared with previous studies, results have been inconsistent regarding differences in microbial community structure based on environmental factors. Therefore, soil bacterial community structures may have more relevance to geographical location than environmental factors (except for extreme conditions)

because the genetic diversity of primitive microbial communities could vary depending on their geographical location.

The aim of this study was to analyze the bacterial diversity on the ecologically important islands of Dokdo. The total number of species identified was 1,239 and 1,055 on Dongdo and Seodo, respectively. By introducing the pyrosequencing technique (distinct from other research methods such as culture-based approaches and DGGE), it was possible to acquire large-scale sequencing data and to identify numerous unculturable bacteria. Therefore, metagenomic analyses are expected to help provide information on bacterial communities and for screening new species in the natural environment of Dokdo.

In summary, to analyze the soil bacterial communities of Dokdo, metagenomic analysis was performed using a pyrosequencing method. The number of OTUs obtained was 1,693 and 1,408 from Dongdo and Seodo, respectively. Statistical analyses (rarefaction curves as well as Chao1, Shannon, and Simpson indices) indicated that bacterial diversity on Dongdo was slightly higher than on Seodo. Most of the validated reads were classified at the phylum level for both the Dongdo and Seodo samples, but the classified reads considerably decreased in number from the phylum level down to the species level. Unlike the higher levels (family and above), the taxonomic composition at the genus level was slightly different between Dongdo and Seodo. The analyses of relative abundance at taxonomic ranks showed variable differences between Dongdo and Seodo. The most important objective of this study was to acquire vast information about the bacterial communities on Dokdo. Through our study, the number of species identified on Dongdo and Seodo was 1,239 and 1,055, respectively. In

conclusion, this study provides key information on the numerous culturable and unculturable bacteria on Dokdo, and is expected to facilitate the screening of new species.

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